SRI International

LP1845 LIQUID GUN PROPELLANT DERMAL TOXICITY STUDIES:

CHO/HGPRT GENE MUTATION ASSAY OF
LP1846 LIQUID GUN PROPELLANT

Final Report

Date: 12 March 1990

By:

Colette J. Rudd, Ph.D., D.A.B.T., Director
Cellular Genetics Program
Study Director

Pamela S. Lee
Cellular Genetics Program
Assistant Study Director

Prepared for:

U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, MD 21701-5012

Project Officer: Robert Finch

Approved for public release; distribution unlimited

Testing Facility:

SRI International
333 Ravenswood Ave.
Menlo Park, CA 94025

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Approved by:

Jon Mirsalis, Ph.D., D.A.B.T., Director
Cellular and Genetic Toxicology Department

Robert M. Sutherland, Ph.D.
Vice President and Executive Director
Life Sciences Division

333 Ravenswood Ave. • Menlo Park, CA 94025
415 326-6200 • TWX 910-373-2046 • Telex 334-486

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The purpose of this study was to evaluate LP1846 Liquid Gun Propellant for toxicity and mutagenic activity at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells. Mutant (HGPRT$^-$) cells were identified by their resistance to 6-thioguanine. LP1846 was evaluated in experiments in the presence and absence of an exogenous metabolic activation system (S9). The cells were treated with culture medium containing the test sample (240 to 1000 µg/ml) for 4 hr. In these experiments, the average cloning efficiencies relative to solvent controls of cultures treated with 1000 µg/ml LP1846 were 61% without S9 and 53% with S9. The criteria for a positive response was a threefold or greater increase in the frequency of 6-thioguanine-resistant colonies, compared with the solvent control cultures, in treated cultures with an average initial survival rate of at least 20%. LP1846 did not induce a concentration-dependent increase in mutant frequency either without or with S9 and was evaluated as negative under both activation conditions.

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FOREWORD

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Colette J Rudd 3/12/90

PI Signature Date

QUALITY ASSURANCE UNIT
Final Report and
Conflict of Interest Statement

The Quality Assurance Unit of SRI International assures that the CHO/HGPRT Gene Mutation Assay of LP1846 Liquid Gun Propellant conducted for U.S.Army Medical Research and Development Command was performed in compliance with Good Laboratory Practices as set forth by the Toxic Substances Control Act, Environmental Protection Agency (CFR 40, Part 792).

Inspections were performed on August 25, 1989 and September 11, 1989 during cloning and counting phases. The Study Director was informed of any findings following inspection and SRI Management was informed August 29, 1989 and September 12, 1989.

Audit of the raw data generated during the conduct of the study and Draft Final Report verification were completed September 19, 1989. The Study Director and SRI Management were notified of audit results on September 20, 1989. The Final Report was reviewed on March 8, 1990 and reaudit was deemed unnecessary. The Final Report accurately describes the methods and Standard Operating Procedures (SOPs) utilized during the study and does reflect the raw data that was generated during the conduct of the study. Any deviations from the protocol or SOPs were made with proper documentation.

This statement certifies that the personnel listed below participated in the inspections and audit of this study. These personnel have not been involved in the generation or evaluation of the data. Participation by the individuals listed below poses no conflict of interest.

Jill E. Kovach Pamela A. Pallakoff

I verify that the above is true to the best of my knowledge.

Jill E Kovach
Quality Assurance Unit

12 March 1990
Date

COMPLIANCE STATEMENT

To the best of our knowledge, the CHO/HGPRT gene mutation assay with LP1846 Liquid Gun Propellant (SRI Study No. 7662-B01-89) was conducted in general conformance with United States Environmental Protection Agency 40 CFR Part 792 Good Laboratory Practice standards with the following exceptions:

1. Test substance characterization and stability data were not developed by SRI International.

2. Assays to verify concentration, stability, and homogeneity of the test substance in the solvent were not performed.

These deviations should not affect the results or conclusions of this study.

Colette J. Rudd

Colette J. Rudd, Ph.D., D.A.B.T.
Study Director

Mar 12, 1990
Date

SUMMARY

The purpose of this study was to evaluate LP1846 Liquid Gun Propellant for toxicity and mutagenic activity at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells. Mutant ($HGPRT^-$) cells were identified by their resistance to 6-thioguanine.

LP1846 was evaluated in experiments in the presence and absence of an exogenous metabolic activation system (S9). The cells were treated with culture medium containing the test sample (240 to 1000 µg/ml) for 4 hr. In these experiments, the average cloning efficiencies relative to solvent controls of cultures treated with 1000 µg/ml LP1846 were 61% without S9 and 53% with S9.

The criteria for a positive response was a threefold or greater increase in the frequency of 6-thioguanine-resistant colonies, compared with the solvent control cultures, in treated cultures with an average initial survival rate of at least 20%. LP1846 did not induce a concentration-dependent increase in mutant frequency either without or with S9 and was evaluated as negative under both activation conditions.

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INTRODUCTION AND PURPOSE

Study Objective

The past decade has seen increasing public concern about the toxicologic risks of exposure to chemicals, particularly the potential genotoxic effects, which may lead to cancer. One of the primary indications of genotoxicity is the ability of a chemical or other agent to induce mutations in DNA. Because carcinogenesis may be initiated by an alteration of one or more genes in mammalian cells, identification of mutagenic agents in the environment is important.

A variety of mammalian cell lines and genetic loci have been used in screening procedures to detect chemically induced mutagenesis. The CHO/HGPRT mutagenesis assay, reviewed by Hsie et al. (1981), is one of the more thoroughly evaluated systems. In this system, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) metabolizes the selection agent 6-thioguanine (TG) to a cytotoxic derivative; cells lacking HGPRT activity are unaffected by the presence of TG. The frequency of $HGPRT^-$ cells can be determined from cloning known numbers of cells in the presence and absence of TG.

Study Purpose

The purpose of this study was to evaluate LP1846 Liquid Gun Propellant for toxicity and mutagenic activity at the HGPRT locus in Chinese hamster ovary (CHO) cells in the absence and presence of a metabolic activation system. Testing for this study was conducted in compliance with Environmental Protection Agency 40 CFR Part 792 Good Laboratory Practice (GLP) standards.

MATERIALS

- Test Article
  - Name: LP1846 Liquid Gun Propellant
  - Supplier: U.S. Army Ballistic Research Laboratory
  - Date material received at SRI International: 3 July 1989
  - Description: Clear liquid
  - Quantity of material received from Sponsor: 20 ml
  - Purity and stability: Identity and purity determined by the Sponsor
  - Storage conditions: Stored at room temperature in original and lightproof secondary containers in the SRI Chemical Repository, Bldg M, Room 217
  - Lot No.: 1846-03.

- Solvent (Negative Control Article)
  - Name: Sterile purified deionized water
  - Source: Millipore Super Q, Building M, Room 212 (No. 123365)

- Positive Control Article for the Assay with Metabolic Activation
  - Name: 3-Methylcholanthrene (MCA; CAS No. 56-49-5)
  - Supplier: Sigma Chemical Co., St. Louis, MO, Catalog No. M6501
  - Date material received at SRI International: 27 June 1988
  - Description: Yellow crystals
  - Quantity of material received from supplier: 1 g
  - Storage conditions: Stored at room temperature in original and secondary lightproof containers
  - Lot No.: 61F-0069.

- Positive Control Article for the Assay without Metabolic Activation

  - Name: Ethyl methanesulfonate (EMS; CAS No. 62-50-0)

  - Supplier: Sigma Chemical Company, St. Louis, MO, Catalog No. M0880

  - Date material received at SRI International: 26 March 1984

  - Description: Clear, colorless liquid

  - Quantity of material received from supplier: 5 g

  - Storage conditions: Stored at 4°C in original and secondary lightproof containers

  - Lot No.: 83F-0279.

- Cell Culture

  - Type of cells: Chinese hamster ovary (CHO) cells

  - Source of cells: American Type Culture Collection (ATCC), Rockville, MD

- S9 Preparation

  - Rats: Male Fischer-344, ~220-255 g

  - Source: Simonsen Laboratories, Inc., 1180C Day Road, Gilroy, CA 95020

  - S9 Lot: IR-46

  - S9 protein concentration: 33 mg/m^1

TEST SYSTEM

Cell Line

The cell line used for the CHO/HGPRT mutation assay was CHO-K1 and was obtained from ATCC. Vials containing the stock cells were stored in a liquid nitrogen freezer. Periodically, a new vial of cells was thawed, and the cells were grown as attached cultures in 75-cm^2 tissue-culture flasks. All cells were incubated at ~37°C in 5% CO_2 at >90% relative humidity.

Media

CHO cells were cultivated in hypoxanthine-free Ham's F12 medium supplemented with 31 µg/ml penicillin (1650 U/mg), 50 µg/ml streptomycin sulfate, and 5% heat-inactivated fetal bovine serum (F12/5). The same medium was used for the chemical exposures without activation; with activation, the final serum concentration was reduced to 0.8%. Cloning medium differed in that it contained 10% fetal bovine serum and 0.22% purified agar (Baltimore Biological Laboratories, Cockeysville, MD). The selective cloning medium contained 30 µM TG.

Metabolic Activation

An Aroclor 1254-induced rat-liver homogenate preparation (S9) was used as the metabolic activation system. Liver enzymes were induced by injecting adult male rats with Aroclor 1254 (500 mg/kg) 5 days before sacrifice. The S9 consisted of a 9000 × g supernatant of liver homogenized in sucrose-phosphate buffer (1 g wet weight of liver to 3 ml of sucrose-phosphate buffer). The protein content was determined for each lot. The S9 was prepared in large lots and stored frozen in liquid nitrogen until use.

The activation mixture (S9-mix) consisted of S9 (5%), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 30 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, and 50 mM sodium phosphate buffer. A ratio of 1 ml of S9-mix to 4 ml of F12/1 per culture was added for the experiments requiring S9, giving a final S9 concentration of 1%.

STUDY DESIGN

Test Dates

Study initiation date: 1 August 1989
Laboratory work initiated: 3 August 1989
Laboratory work completed: 15 September 1989
Study completion date: Submission of final report.

Dilution of Test Article

The test article was diluted in sterile water to form a series of 200X stock solutions that, when diluted in the culture medium, resulted in the desired test concentrations.

Controls

The positive control chemicals were 200 µg/ml EMS, which induces mutagenesis without metabolic activation, and 5 µg/ml MCA, which induces mutagenesis only with metabolic activation. For assays with metabolic activation, the activation mixture was also added to the solvent control cultures. The stability of the control substances was not measured directly; however, an indirect evaluation was based on the acceptability of the mutation frequencies of cells exposed to solvent control and positive control substances (see "Criteria for Acceptability" below).

Preliminary Cytotoxicity Experiments

Preliminary experiments on the test article were performed in the absence and presence of metabolic activation, using a single culture for each concentration tested. The maximum concentration was 1000 µg/ml. The results of this experiment were used to select concentrations to be tested in the mutagenesis experiments.

Mutagenesis Experiments

For each mutagenesis experiment, five concentrations of the test article were selected. Duplicate cultures were used for each test article dilution and for the positive controls; three cultures were used for the solvent controls. The experiments were performed according to the detailed procedure described below.

Procedural Detail

Cell preparation. Approximately 24 hr prior to chemical treatment, 2 $\times$ 10^6 exponentially growing cells were seeded into 75-cm^2 tissue-culture flasks in 20 ml of F12/5 medium. The cells derived from each one of these flasks are referred to as a "culture." Immediately before exposure, the cells were observed using an inverted microscope to check for cell attachment and estimate the level of confluence of the monolayer.

Chemical exposure without metabolic activation. The medium in each flask was replaced by 5 ml of F12/5. The appropriate chemical stock solution was added, and the cells were incubated for 4 hr. To terminate the exposure period, the treatment solutions were removed, and the cells were washed twice with 10 ml of Puck's saline and subcultured for cytotoxicity determination and phenotypic expression as described below.

Chemical exposure with S9 metabolic activation. The F12/5 medium in each flask was replaced by 4 ml of F12/1 and 1 ml of the activation mix previously described. The appropriate chemical stock solution was added, and the cells were incubated for 4 hr. The exposure period was terminated as described for the nonactivated cultures.

Determination of cytotoxic effects of test articles. At the end of the chemical exposure period, the cells in each culture were detached from the flask with 0.02% trypsin-EDTA and suspended in 10 to 20 ml of F12/5. A Coulter® counter was used to determine the cell number, and an aliquot containing at least 1 to 2 $\times$ 10^6 cells was added to 20 ml of F12/5 in a 75-cm^2 or larger tissue-culture flask for phenotypic expression (described below). A serial dilution was performed from the cell suspension so that

~500 cells were plated into two 100-mm petri dishes in approximately 35 ml of cloning medium per dish. After incubation for at least 14 days, the resulting cell colonies were counted using an automatic colony counter with a standard 50-mm lens. For evaluation purposes, survival was expressed as the cloning efficiency relative to the control cells, calculated by the formula: (average cloning efficiency of treated cells ÷ average cloning efficiency of control cultures) x 100.

Phenotypic expression of induced mutants. The cultures were incubated for 7 days for expression of any mutations. Cells were subcultured every 2 to 3 days during this period by trypsinizing the flasks, counting the cells, and replating 2×10^6 cells.

Selection of TG-resistant (TG^r) cells. After the expression period, approximately 3×10^6 cells from each culture were seeded in 100 ml of cloning medium supplemented with TG for selection of TG^r cells. Approximately 600 cells were seeded in 100 ml of nonselective cloning medium to determine the percentage of viable unselected cells. The cell suspensions from each of those conditions were then poured into three 100-mm petri dishes. After incubation of the cells for at least 14 days, the cell colonies in each petri dish were counted either with an automatic colony counter with a standard 50-mm lens or by hand.

Determination of mutant frequency. The mutant frequency of each culture (the ratio of the number of mutant cells to the number of wild-type cells) was calculated by dividing the number of TG^r colonies by 5,000 times the number of unselected (viable) colonies to compensate for the difference in the number of cells cloned initially and the absolute cloning efficiency of the cells.

Data Analysis

Criteria for Acceptability of Mutagenesis Experiments

Experiments meeting these criteria were evaluated for mutagenic response, and experiments that did not meet the criteria were repeated. However, because the mutagenesis assay is a complex biological system, the criteria are used as guidelines, and exceptions may be justified.

Negative controls. The average mutant frequency in an experiment should be less than 60 TG^r colonies per 10^6 viable cells.

Positive controls. The average mutant frequencies should be at least three times greater than the average mutant frequencies of the solvent controls. If the relative cloning efficiency is less than 60%, then the average mutant frequency of the positive control cultures should be fivefold that of the solvent controls.

Criteria for Evaluating Results of Acceptable Mutagenesis Experiments

Positive. The test results for a particular test article are considered positive if a dose-related increase in the number of mutant colonies occurs and the mutant frequencies of duplicate cultures (with an average initial survival rate of at least 20%) treated with one or more concentrations of the test article are at least three times the average of those from the solvent control cultures.

Negative. The results are considered negative if the test article does not induce a response according to the above criteria.

No statistical analyses were performed for this study.

Records

All raw data (including those on culture preparation, chemical exposure, chemical removal, cell density determination, cloning, and colony-counting forms and data tables affixed into SRI notebooks), documentation, protocols, correspondence, and final reports will be retained at SRI International, Menlo Park, CA, in the Records Center for no less than ten years. Wet specimens (e.g., colonies in soft agar) and samples of the control articles have not been retained. Any remaining unused test material will be returned to the Sponsor upon completion of the study.

Key Personnel

Technical assistance for this study was contributed by Kathryn D. Suing, Cell Biologist; Daniel C. Blachman, Cell Biologist; and Glenn D. Cunningham, Biological Technician.

RESULTS AND DISCUSSION

The study to investigate the toxic and the mutagenic potential of LP1846 Liquid Gun Propellant in the CHO/HGPRT gene mutation assay was conducted according to the protocol "Chinese hamster ovary (CHO) HGPRT gene mutation assay." Results of the experiments used to evaluate the potential mutagenicity of LP1846 are presented in Tables 1 and 2.

For this project, one experiment without S9 compared the effect of LP1846 with the positive control chemical EMS (200 µg/ml). Another experiment was performed with S9, comparing the effect of LP1846 with the positive control chemical MCA (5 µg/ml). EMS increased the average mutant frequency 5.8-fold (to 180 mutants per 10^6 cells). With MCA and S9, a 5.7-fold increase in average mutant frequency (to 137 mutants per 10^6 cells) was induced.

Cytotoxicity

The toxicity of LP1846 was measured by comparing the cloning efficiency of the treated cells with that of the solvent control cultures immediately after the exposure period. In the preliminary cytotoxicity experiment (data not included), the cells were treated with 13 concentrations of LP1846 (from 0.25 to 1000 µg/ml), without and with S9. Test concentrations were selected to include five concentrations from 240 to 1000 µg/ml, the maximum concentration specified by the protocol. In the mutagenesis experiments the relative cloning efficiencies of cells treated with 1000 µg/ml were 61% without S9 and 53% with S9.

Mutagenicity

LP1846 did not induce a mutagenic response (threefold or greater increase in the average frequency of TG^r mutant cells) in the experiments with or without S9. Without S9 (Table 1), increases in mutant frequency were 1.2 and 1.0 times that of the average solvent control value at 700 and 1000 µg/ml, respectively. With S9 (Table 2), the average mutant

frequencies of the treated cultures ranged from 1.4 to 1.8 times that of the solvent control cultures. Therefore, LP1846 was evaluated as negative without and with activation.

Table 1

CHO/HGPRT MUTAGENESIS EXPERIMENT A2:
EVALUATION OF LP1846 WITHOUT ACTIVATION

Chemical	Concentration	Cytotoxicity (%CE*)	Average RCE† (%)	Total Unselected Colonies	Total TG^r Colonies	Mutant Frequency (per 10^6 cells)
Water	5 µl/ml§	85 75 80 (80)#	100	394 457 362 (404)	70 51 66 (62)	36 22 36 (31)
LP1846	240 µg/ml	72 85 (79)	99	417 411 (414)	73 52 (63)	35 25 (30)
	343	69 65 (67)	84	346 405 (376)	21 59 (40)	12 29 (21)
	490	61 71 (66)	83	362 394 (378)	43 53 (48)	24 27 (26)
	700	57 51 (54)	68	272 352 (312)	59 55 (57)	43 31 (37)
	1000	45 52 (49)	61	314 362 (338)	37 68 (53)	24 38 (31)
EMS	200 µg/ml	55 54 (55)	69	227 304 (266)	228 242 (235)	201 159 (180)

*Cloning efficiency of cells after chemical treatment, assuming 500 cells plated.

†Cloning efficiency relative to the average solvent control value.

§Equivalent to volume of stock test article solution added to each culture.

#Numbers in parentheses represent average values.

Table 2

CHO/HGPRT MUTAGENESIS EXPERIMENT A3:
EVALUATION OF LP1846 WITH ACTIVATION

Chemical	Concentration	Cytotoxicity (%CE*)	Average RCE† (%)	Total Unselected Colonies	Total TG^r Colonies	Mutant Frequency (per 10^6 cells)
Water	5 µl/ml§	69 58 83 (70)#	100	320 283 321 (308)	54 30 26 (37)	34 21 16 (24)
LP1846	240 µg/ml	59 57 (58)	83	368** 297 (333)	81 42 (62)	44 28 (36)
	343	39 58 (49)	70	339 329 (334)	51 61 (56)	30 37 (34)
	490	49 50 (50)	71	340 267 (304)	84 45 (65)	49 34 (42)
	700	33 47 (40)	57	282 286 (284)	38 66 (52)	27 46 (37)
	1000	33 41 (37)	53	330 340 (335)	64 60 (62)	39 35 (37)
MCA	5 µg/ml	83 75 (79)	113	297 249 (273)	188 183 (186)	127 147 (137)

*Cloning efficiency of cells after chemical treatment, assuming 500 cells plated.

†Cloning efficiency relative to the average solvent control value.

§Equivalent to volume of stock test article solution added to each culture.

#Numbers in parentheses represent average values.

**One of three culture plates was contaminated with mold. The number of colonies on this plate was calculated as the average of the remaining two plates.

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